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# Comparison of Heterophil Phagocytosis for Heterophil- Adapted *Salmonella enteritidis* (HASE) and Wild-Type *Salmonella enteritidis* (SE)

## Abstract

Serial passage of *Salmonella enteritidis* (SE) yields heterophil-adapted SE (HASE) strains that have resulted in decreased shedding of SE in feces and reduced egg contamination. Additionally, increasing the number of heterophil passages further reduced the number and frequency of fecal shedding. To evaluate SE and heterophil interaction, nine SE strains were fluorescein isothiocyanate-labeled and were viable. There were six wild-types: SE TK 474, SE TK 584, SE TK 599, SE TK 600, SE TK 655, and SE TK 657; and three HASE strains: TK 499 heterophil adapted five times, TK 598 heterophil adapted six times, and TK 605 heterophil adapted 11 times. Trials were repeated seven times in duplicate with heterophils isolated from seven healthy chickens. Heterophils were incubated with the bacterial strains at 41 °C for 15 min, and 10,000 heterophils were analyzed by flow cytometry. Percentage of phagocytosis and mean channel number of fluorescence were compared. Both parameters were significantly increased for all HASE-type strains compared with wild-type, nonadapted SE strains. Increased phagocytosis of HASE bacterial strains may be significant in processing and elimination of the HASE strains and may be related to the protective effect of HASE by decreased shedding of wild-type SE challenge strains.

## Keywords

Veterinary Pathology, Veterinary Microbiology and Preventive Medicine

## Disciplines

Large or Food Animal and Equine Medicine | Veterinary Microbiology and Immunobiology | Veterinary Pathology and Pathobiology

## Comments

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*Research Note*—

## Comparison of Heterophil Phagocytosis for Heterophil-Adapted *Salmonella enteritidis* (HASE) and Wild-Type *Salmonella enteritidis* (SE)

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**SUMMARY.** Serial passage of *Salmonella enteritidis* (SE) yields heterophil-adapted SE (HASE) strains that have resulted in decreased shedding of SE in feces and reduced egg contamination. Additionally, increasing the number of heterophil passages further reduced the number and frequency of fecal shedding. To evaluate SE and heterophil interaction, nine SE strains were fluorescein isothiocyanate-labeled when viable. There were six wild-types: SE TK 474, SE TK 584, SE TK 599, SE TK 600, SE TK 655, and SE TK 657; and three HASE strains: TK 499 heterophil adapted five times, TK 598 heterophil adapted six times, and TK 605 heterophil adapted 11 times. Trials were repeated seven times in duplicate with heterophils isolated from seven healthy chickens. Heterophils were incubated with the bacterial strains at 41 C for 15 min, and 10,000 heterophils were analyzed by flow cytometry. Percentage of phagocytosis and mean channel number of fluorescence were compared. Both parameters were significantly increased for all HASE-type strains compared with wild-type, nonadapted SE strains. Increased phagocytosis of HASE bacterial strains may be significant in processing and elimination of the HASE strains and may be related to the protective effect of HASE by decreased shedding of wild-type SE challenge strains.

**RESUMEN.** *Nota de Investigación*—Comparación de la fagocitosis por heterófilos contra *Salmonella enteritidis* adaptada a heterófilos (HASE) y *Salmonella enteritidis* original.

Se obtuvieron cepas de *Salmonella enteritidis* adaptadas a heterófilos mediante pasajes seriados en cultivos de heterófilos, resultando en la disminución de la eliminación de *S. enteritidis* a través de la materia fecal y la reducción en la contaminación de los huevos. Además, el aumento en el número de pases en cultivos de heterófilos redujo aún más el número y la frecuencia de la contaminación fecal. Para evaluar la interacción de *S. enteritidis* y de los heterófilos se marcaron nueve cepas de *S. enteritidis* en su forma viable con isotiocianato de fluoresceína: las cepas originales SE TK 474, SE TK 584, SE TK 599, SE TK 600, SE TK 655, SE TK 657 y las cepas adaptadas a cultivos de heterófilos: TK 499 (5 pases), TK 598 (6 pases) y TK 605 (11 pases). Los experimentos fueron repetidos siete veces en duplicado con heterófilos aislados de siete pollos sanos. Los heterófilos fueron incubados con las cepas bacterianas a 41 C durante 15 minutos y se analizaron diez mil heterófilos mediante la citometría de flujo. Se comparó el porcentaje de fagocitosis y el número promedio de canal de fluorescencia. Los dos parámetros aumentaron significativamente para todas las cepas adaptadas a cultivos de heterófilos comparadas con las cepas de *S. enteritidis* originales. El aumento de la fagocitosis de las cepas adaptadas a cultivos de heterófilos puede ser significativo en el proceso y eliminación de estas cepas y puede estar relacionado al efecto protector de las cepas adaptas a heterófilos por la disminución en la diseminación de las cepas originales de *S. enteritidis* de desafío.

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Key words: heterophil phagocytosis, flow cytometry, *Salmonella enteritidis*, heterophil adaptation

Abbreviations: FITC = fluorescein isothiocyanate; HASE = heterophil-adapted *Salmonella enteritidis*; HBSS = Hanks balanced salt solution; MnX = mean channel number; SE = *Salmonella enteritidis*

Salmonellosis caused by *Salmonella enteritidis* (SE) from eggs is a leading cause of foodborne illness (4). SE may be transmitted from the infected ovaries and lower reproductive tract to the egg with the hen serving as an inapparent carrier. There is a need to decrease fecal shedding of SE and prevent SE contamination of eggs. In order to create a vaccine for *Salmonella*, strong mucosal immunity must be established, and usually only attenuated live vaccines are effective against *Salmonella* (8). An efficacious oral vaccine (SC-54, NOBL®) was created with clones of *Salmonella choleraesuis* passaged through swine neutrophils (12,13). A similar technique has been used to develop an oral SE vaccine with heterophil-adapted SE (HASE). Fecal shedding of HASE occurred for a maximum of 6 days in trials with various HASE-exposed hens *vs.* 40 days or longer in SE-challenged control hens (10,11). When HASE-treated hens were challenged with wild-type SE strains, the wild-type SE was shed in 2.8% of fecal samples and shedding stopped on day 9 postchallenge, compared with 28.1% positive fecal samples and persistent shedding in challenge control hens (11). Egg production was not affected by HASE oral vaccination, and HASE was not isolated from any of 1019 eggs laid by HASE-exposed hens. Hens first given an oral HASE vaccine and then challenged with wild-type SE did not transmit SE to eggs (10,11). In contrast, wild-type SE strains were transmitted to eggs at rates ranging from 5.6% to 9.2%.

In previous trials, avian SE were fluorescein isothiocyanate (FITC) labeled as live and heat-killed bacteria and compared for percentage of heterophil phagocytosis, phagocytic index, and mean channel number (MnX) fluorescence. Wild-type pathogenic SE TK 474 and SE TK 599 strains and HASE TK 499 (heterophil adapted five times [5× adapted]) and HASE TK 605 (11× adapted) strains were compared. HASE TK 605 (11× adapted) had a significantly higher percentage of phagocytosis and phagocytic index than the other bacterial strains

(3). The live-labeled FITC bacteria had an increased phagocytic index compared with heat-killed strains (3). In preliminary assays of microtiter plate counts of cell-associated and phagocytosed bacteria, the mean percentage of HASE TK 605 (11× adapted) was 82% *vs.* a lower 46% association of wild-type pathogenic SE TK 474 bacteria (J. K. Akunda, unpubl. data).

Granulocyte (heterophil) attenuation of *Salmonella* virulence involves an adaptive process induced by exposure of SE to heterophils. Because phagocytosis is a major function of heterophils, this study was conducted to further investigate the SE bacteria and heterophil interaction and document differences between wild-type SE strains and HASE strains.

## MATERIALS AND METHODS

**Experimental design.** The number of trials needed was statistically determined by power analysis from preliminary data. Heterophils were collected from 6-to-8-wk-old nonvaccinated Arbor Acres broiler chickens and incubated with three HASE strains and six wild-type SE strains that had been FITC labeled while viable. FITC labeling was evaluated by fluorescent microscopy and flow cytometry to verify uniform labeling, and viability was assessed by plate counts. Bacteria were opsonized with 5% pooled serum at room temperature for 5 min. Heterophils were isolated and incubated with opsonized FITC-labeled bacteria for 15 min at 41 C. Samples were run in duplicate and seven trials were performed. Samples consisted of isolated heterophils, non-trypan blue-quenched samples, samples quenched with trypan blue, nonphagocytosing heterophils, and FITC-labeled bacteria. The samples were analyzed by flow cytometry for percentage of phagocytosis, indicating the number of heterophils that ingest FITC-labeled bacteria, and MnX of fluorescent intensity that corresponds to the number of fluorescent bacterial particles that have been ingested. Increased channel numbers indicate an increased fluorescent signal that correlates with the number of fluorescent bacteria within cells. Heterophil nonspecific autofluorescence was excluded by setting the detection above the background level. Samples were analyzed for sig-

nificance and differences were located with t-tests and Tukey's Studentized range distribution ( $P < 0.05$ ).

**Bacteria and FITC labeling.** The following wild-type SE bacteria were used for FITC labeling: SE TK 474 (phage type 8, original isolate used for HASE strains), SE TK 600 (phage type 8, University of Minnesota) (5), SE TK 584 (phage type 8, nalidixic acid resistant, Iowa State University), SE TK 599 (phage type 8, passaged TK 584 through chicken), SE TK 655 (phage type 13, University of Pennsylvania), and SE TK 657 (phage type 4, University of Pennsylvania). HASE strains used were heterophil-adapted strains serially passaged from SE TK 474 including HASE TK 499 (5 $\times$  adapted), HASE TK 598 (6 $\times$  adapted), and HASE TK 605 (11 $\times$  adapted).

FITC labeling was done by modification of a previously described method (1,7). For FITC labeling of live bacteria, the heat inactivation step was eliminated, and procedures were conducted on ice and in a 4 C centrifuge to prevent continued bacterial growth. The live FITC-labeled bacteria were suspended in 10 ml Hanks balanced salt solution (HBSS) with 50% glycerol, aliquoted into 0.5 ml, flash frozen, and stored at  $-70^{\circ}\text{C}$ . Bacterial titer plate counts were done before and after FITC labeling and viability was  $\geq 85\%$ . FITC-labeled bacterial counts were calculated by adding a known ratio of fluorescent beads during flow cytometric analysis. Additionally, peak MnX fluorescence appeared as a consistent single peak, indicating uniform FITC labeling of bacteria.

**Heterophils.** Heterophils were isolated from seven 6-to-8-wk-old nonvaccinated Arbor Acres broiler chickens by a slight modification of a previously described technique (1,2). Twelve to 24 ml of blood was collected, maintaining previously described proportions, and the lysing step was eliminated because of interference of cell remnants with flow cytometry. Cells were diluted in HBSS to  $1 \times 10^6/\text{ml}$ .

**Flow cytometric phagocytosis assay.** Duplicate samples were used in each test for all bacterial strains in seven trials with slight modifications of previously described techniques (1). All bacterial strains were opsonized with 5% pooled chicken serum with a wild-type SE agglutinin titer of 1:8 and a titer of 1:2 against HASE TK 605 (11 $\times$  adapted). Then  $2 \times 10^7$  colony-forming units/ml bacteria and heterophils at  $1 \times 10^6/\text{ml}$ , in a volume of 1 ml, were centrifuged for 10 min at  $25 \times g$  for contact, resuspended, and incubated at  $41^{\circ}\text{C}$  for 15 min in a shaker water bath on slow rotation. One hundred microliters of 40 mM ethylenediaminetetraacetic acid was added to each tube to stop phagocytosis, and tubes were placed on ice. One hundred microliters of 0.4% trypan blue in HBSS at  $4^{\circ}\text{C}$  was added to the quenched samples and incubated on ice for 15 min. Samples were centrifuged twice with 0.9% sterile saline for 15 min at  $250 \times g$  and supernatants were

discarded. Samples then were reconstituted to the original volume of 1 ml by fixation in 1% paraformaldehyde in the dark for 20 min (1). The samples were again centrifuged for 15 min at  $250 \times g$  and, after supernatants were removed, reconstituted to 1 ml with HBSS, refrigerated, and analyzed within 48 hr. Paired samples were used for nonquenched and quenched (to obscure surface bacteria) phagocytic studies. Samples run for each bacterial strain consisted of 1) heterophils only, 2) nonquenched samples (adherent and phagocytosed bacteria with heterophils), 3) quenched samples (phagocytosed bacteria in heterophils), 4) nonphagocytosing heterophils at  $3^{\circ}\text{C}$  on ice, and 5) FITC-labeled bacteria. Ten thousand heterophils were analyzed by a Coulter Epics XL with Coulter software (Coulter Electronics, Hialeah, FL) as previously described (1). Percentage of phagocytosis and MnX of fluorescence were determined for the heterophil population. Negative control phagocytosis ranged from 0.4% to 1.1%, indicating nonspecific bacterial adherence.

**Statistical analysis.** The number of trials needed was statistically determined by power analysis from preliminary data. Also, a multivariate MANOVA test was used as a screening test to evaluate if bacterial strains were affecting the MnX and phagocytosis jointly ( $P < 0.0001$ ). Analysis of variance (ANOVA) was performed, significant differences were located, and pairwise comparisons were made between all bacterial strains with a t-test and Tukey's Studentized range distribution to control for type I error ( $P < 0.05$ ).

## RESULTS AND DISCUSSION

Because determining colony plate count phagocytosis can be problematic when trying to remove adherent bacteria by centrifugation or addition of antibiotics that might be transported into the cell and artifactually alter the percentage of phagocytosis, flow cytometry with trypan blue quenching was used to evaluate phagocytosis (6,9). Trypan blue appeared to adequately mask adherent bacteria because quenched samples had decreased MnX and phagocytosis compared with nonquenched samples by flow cytometry (Table 1) and fluorescent microscopy. The live FITC labeling of bacteria was elected because heat killing could alter antigenicity by denaturing membrane proteins or other bacterial proteins and to maximize phagocytosis, as demonstrated in earlier studies (3).

The MANOVA test indicated the type of bacterial strain had a highly significant effect ( $P < 0.0001$ ) on percentage of phagocytosis and



Table 1. Flow cytometric analysis of mean and standard deviation of mean channel number (MnX), indicating fluorescent intensity that corresponds to the number of fluorescent bacterial particles that have been ingested, and percentage of phagocytosis, indicating the number of heterophils that ingest FITC-labeled bacteria, for nonquenched and trypan blue-quenched samples for all HASE samples and all wild-type SE samples.<sup>A</sup>

	HASE	Wild-type SE
Nonquenched MnX	12.75 ± 2.85 <sup>a</sup>	6.05 ± 0.74 <sup>b</sup>
Quenched MnX	5.56 ± 1.21 <sup>a</sup>	3.00 ± 0.27 <sup>b</sup>
Nonquenched percentage of phagocytosis	42.59 ± 4.61 <sup>a</sup>	34.62 ± 1.80 <sup>b</sup>
Quenched percentage of phagocytosis	37.19 ± 0.85 <sup>a</sup>	28.69 ± 2.81 <sup>b</sup>

<sup>A</sup>Different lowercase superscripts in the same row indicate significant differences with the use of Tukey's Studentized range distribution ( $P < 0.05$ ).

MnX. Comparisons with Tukey's Studentized range and t-tests ( $P < 0.05$ ) identified the same significant differences between bacterial strains. In all bacterial strain comparisons, HASE bacteria MnX values were significantly higher ( $P < 0.05$ ) than those of wild-type SE nonadapted strains for pairwise comparisons, and the mean MnX was significantly higher ( $P < 0.05$ ) for all HASE strains compared with wild-type SE strains (Table 1). The mean percentage of phagocytosis was significantly higher ( $P < 0.05$ ) for HASE strains, but percentage of phagocytosis was not significantly higher in all pairwise statistical comparisons because of the variation in percentage of phagocytosis within bacterial strains. Three of the four highest percentage of phagocytosis values were HASE strains. No significant differences were present for MnX or percentage of phagocytosis for any wild-type SE strains. Therefore, HASE strains had significantly increased MnX values and an increased mean percentage of phagocytosis when compared with wild-type SE strains (Table 1). The MnX values for HASE strains were approximately double the values for wild-type SE, and these findings also were present in preliminary data (3). Overall, HASE bacteria had increased uptake by heterophils compared with wild-type SE strains.

Significant differences were detected between HASE TK 605 (11× adapted) when compared with HASE TK 499 (5× adapted) and HASE TK 598 (6× adapted), but significant differences were not present between HASE TK 499 (5× adapted) and HASE TK 598 (6× adapted). In *in vivo* previous studies, HASE TK 605 (11× adapted) resulted in less fecal shedding and egg contamination when compared with the HASE TK 598 (6× adapted) strain (11);

therefore, increased passage of SE through heterophils appeared more effective in preventing shedding. It is of interest to note that two HASE strains, TK 499 (5× adapted) and TK 598 (6× adapted), with a single difference in heterophil adaptation rates, were significantly different from the HASE TK 605 (11× adapted) for MnX.

Previous work indicates that HASE TK 605 (11× adapted) strain would be the best candidate to prevent fecal and egg contamination by SE (10,11). Increased phagocytosis of HASE strains, especially HASE TK 605 (11× adapted), compared with wild-type strains has been repeatable. Possibly the increased phagocytosis of HASE-strain bacteria by heterophils is the reason that HASE strains are less virulent because they are more readily recognized by the heterophil and processed for recognition and eliminated *vs.* more pathogenic strains that may have decreased phagocytosis and decreased elimination from the host. This possibility assumes that the interaction of the heterophils within tissues is similar to the peripheral blood-derived heterophils. How increased phagocytosis of HASE strains *vs.* non-HASE wild-type strains relates to the ability of HASE strains to decrease shedding of wild-type SE challenge strains is currently unknown. The avirulent HASE strains may enable the heterophils to more effectively eliminate wild-type SE challenge strains. Further studies are needed to characterize the mechanism that underlies the effectiveness of HASE strains.

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